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(54) Title: METHOD FOR PREVENTING ENDOTHELIUM DAMAGE IN MAMMALS AND FOR ALLEVIATING PAIN ASSOCIATED WITH GOUT AND ARTHRITIS

(57) Abstract

A method for preventing endothelium damage in mammals, particularly for the treatment of cardiovascular disorders associated to diabetes and uraemia. The invention also relates to a method for alleviating pain associated with gout and arthritis in mammals. The method comprises administering to a mammal an effective amount of an SSAO inhibitor to block the formation of formaldehyde in the endothelium or cartilage tissues for the purpose of treating cardiovascular disorders associated to diabetes and uraemia or for alleviating pain associated with gout and arthritis.

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TITLE OF THE INVENTION

Method for preventing endothelium damage in mammals and for alleviating pain associated with gout and arthritis.

BACKGROUND OF THE INVENTION

Conventional treatment with diet, insulin, and oral hypoglycaemic agents has been quite successful in ameliorating insulin deficiency and prolonging life. Still, the development of chronic complications affecting the vascular and nervous systems has been difficult to avoid. In fact, damage to the endothelium is widely observed in diabetes, atherosclerosis and hypertension. The mechanism(s) of such serious deterioration is not well understood.

SUMMARY OF THE INVENTION

In accordance with the present invention, there is provided a method for preventing endothelium damage in mammals. The method comprises administering to a patient in need thereof an effective amount of a SSAO inhibitor for the purpose of preventing endothelium damage in mammals. As a preferred feature of the present invention, there is provided a method for the treatment of cardiovascular disorders associated with diabetes and uraemia in mammals. The method comprises administering to a patient in need thereof an effective amount of a SSAO inhibitor for the purpose of treating cardiovascular disorders associated to diabetes and uraemia. The invention also relates to a method for alleviating pain associated with gout and arthritis in mammals. The method comprises administering to a patient in need thereof an effective amount of a SSAO inhibitor for the purpose of alleviating pain associated with gout and arthritis in mammals.

Preferred SSAO inhibitors include (E)-2-(4-fluorophenethyl)-3-fluoroallylamine HCl (MDL-72974A) and derivatives of fluoroallylamine, semicarbazide and

other hydrazide. Compounds such as those described in Lyles et al. (1987 Biochem. Pharmacol. 36:2847 and in U.S. Patent 4,650,907 issued March 7, 1987, U.S. Patent 4,916,151 issued April 10, 1990, U.S. Patent 4,943,593 issued July 24, 1990, U.S. Patent 4,965,288 issued October 23, 1990, U.S. Patent 5,021,456 issued June 4, 1991, U.S. Patent 5,059,714 issued October 22, 1991, U.S. Patent 4,699,928 issued October 13, 1987, European patent application 295604 of December 21, 1988, European patent application 224924 of June 10, 1987 and European patent application 168013 of January 15, 1986, hereby incorporated by reference can be used in the context of the present invention.

Also within the scope of the present invention is the use of SSAO inhibitors for preventing endothelium damage in mammals. A preferred embodiment of the invention includes the use of the SSAO inhibitor in the treatment of cardiovascular disorders associated with diabetes and uraemia in mammals. Also within the scope of the invention is the use of SSAO inhibitors for alleviating pain associated with gout and arthritis in mammals. Preferred SSAO inhibitors include those referred to above.

Also within the scope of the present invention is the use of a SSAO inhibitor in the preparation of a medicament for preventing endothelium damage in mammals. A preferred embodiment of the present invention includes the use of a SSAO inhibitor for the preparation of a medicament for the treatment of cardiovascular disorders associated with diabetes and uraemia in mammals. The invention also relates to the use of a SSAO inhibitor in the preparation of a medicament for alleviating pain associated with gout and arthritis in mammals. Preferred SSAO inhibitors include those referred to above.

The present invention also relates to a method for the *in vivo* inhibition of SSAO. The method

comprises administering to a patient in need thereof a SSAO inhibitory substance or a pharmaceutically acceptable salt thereof in sufficient concentration to provide the desired level of SSAO inhibition. Preferred SSAO inhibitors include those referred to above.

The present invention is also concerned with the use of SSAO inhibitors for the in vivo inhibition of SSAO. Preferred SSAO inhibitors include those referred to above.

Vascular damage is a major contributing factor to morbidity and mortality in diabetes. Damage to the endothelium thus appears to be a target in diabetes, atherosclerosis and even in hypertension. SSAO inhibitor treatment of patients afflicted with these disorders is therefore a choice therapy to reduce endothelium damage.

Arthritis and gout result in damage of cartilage tissues. This damage causes the release of SSAOs located in cartilage tissues. The deamination of methylamine to formaldehyde by SSAOs results in formaldehyde interacting with nerve cells. This interaction is responsible for the pain experienced by patients suffering from gout and arthritis. Inhibition of SSAOs by appropriate inhibitors alleviates this pain by preventing deamination of methylamine to formaldehyde.

Retinopathy is more prevalent in diabetes than in the normal population (Klein et al, 1989, Arch. Ophthalmol. 107:237). Relatively large amount of SSAO activity has been detected in the rat eye (Cao Danh H, et al,1985, J. Pharm. Pharmacol 37:354). It is possible that bio-conversion of methylamine to formaldehyde may be enhanced in the microvessels, the retina or the smooth muscles of the eye in diabetics. As a result the eye will be damaged and subsequently causes blindness in these patients. Methanol is well

known to cause blindness. The mechanism of such damage is also known to be related to the formation of formaldehyde by enzymes. Inhibition of SSAO by appropriate inhibitors can therefore contribute in reducing incidence of retinopathy in diabetes patients.

DETAILED DESCRIPTION OF THE INVENTION

The invention relates to a method for preventing endothelium damage in mammals, particularly the treatment of cardiovascular disorders associated with diabetes and uraemia using SSAO inhibitors. The invention also relates to a method for alleviating pain associated with gout and arthritis using SSAO inhibitors.

SSAO is an enzyme or group of enzymes residing predominantly in the plasma membrane of vascular smooth muscle cells, such as in blood vessels and heart tissues. Although this enzyme has been known for quite some time, its physiological importance had not been well established.

Methylamine is deaminated by several semicarbazide-sensitive amine oxidases (SSAO) prepared from blood and vascular tissues of several different species including human. While methylamine itself is relatively nontoxic towards endothelial cells obtained from both human vein artery and calf pulmonary veins, it becomes very toxic in the presence of SSAO. It has been found that the endogenous bio-conversion of methylamine to formaldehyde is responsible for certain pathological conditions such as cardiovascular damage. This endogenous bio-conversion can be stopped in mammals by administering suitable amounts of SSAO inhibitors.

SSAO inhibitors, i.e. MDL-72974A (E)-2-(4-fluorophenethyl)-3-fluoroallylamine HCl) and semicarbazide, effectively protected cells from SSAO-methylamine induced damage. This shows that the cytotoxicity of methylamine is a consequence of its

deamination. Formaldehyde, the deaminated product of methylamine, was found to be responsible for toxic effects in cells. Hence, an abnormal metabolism of methylamine is likely to be involved in endothelial injury by inducing atherosclerotic plaque formation and thus contributing in causing the cardiovascular disorders seen in diabetes. Blocking the production of formaldehyde by inhibition of SSAO activity (i.e. by SSAO inhibitors, MDL-72974A and semicarbazide) prevents the deterioration of the cardiovascular tissues in disorders such as diabetes, uraemia, and alleviates pain associated with gout and arthritis.

The dosages of SSAO inhibitors required to efficiently prevent endothelium damage in mammals, particularly to treat cardiovascular disorders in diabetes and uraemia patients or to alleviate pain associated with gout and arthritis vary depending upon the severity of the ailment.

The SSAO inhibitors can be administered in various manners to achieve the desired SSAO inhibitory effect. The SSAO inhibitors can be administered alone or in combination with pharmaceutically acceptable carriers or diluents, the proportion and nature of which are determined by the solubility and chemical properties of the inhibitor selected, the chosen route of administration, and standard pharmaceutical practice. The SSAO inhibitors may be administered orally in solid dosage forms, e.g. capsules, tablets, powders, or in liquid forms, e.g. solutions or suspensions. The inhibitors may also be injected parenterally in the form of sterile solutions or suspensions. Solid oral forms may contain conventional excipients, for instance: lactose, sucrose, magnesium stearate, resins, and like materials. Liquid oral forms may contain various flavoring, coloring, preserving, stabilizing, solubilizing, or suspending agents. Parenteral preparations are sterile aqueous or

non-aqueous solutions or suspensions which may contain certain various preserving, stabilizing, buffering, solubilizing, or suspending agents. If desired, additives, such as saline or glucose may be added to make the solutions isotonic.

The amount of SSAO inhibitor administered can vary and can be any effective amount. Unit doses of these inhibitors can contain, for example, from about 0.1 mg to about 100 mg of the inhibitors and may be administered, for example, one or more times daily, as needed.

The term "unit dosage form" is used herein to mean a single or multiple dose form containing a quantity of the active ingredient in admixture with or otherwise in association with the diluent or carrier, said quantity being such that one or more predetermined units are normally required for a single therapeutic administration. In the case of multiple dose forms such as liquids or scored tablets, said predetermined unit is one fraction such as 5 ml (teaspoon) quantity of a liquid or a half or quarter of a scored tablet, of the multiple dose form.

RESEARCH DESIGN AND METHODS

SSAO was prepared from human umbilical arteries. The dissected tissues were homogenized with a Polytron homogenizer in chilled 0.01 M phosphate buffer (pH 6.8). These crude homogenates were then centrifuged at 800 g for 10 min and the supernatants centrifuged further at 32,000 g for 30 min. These final supernatant enzyme preparations were either used immediately or stored at -70°C. In the study of SSAO on cell survival the enzyme preparation was thawed and sterilized through a 0.22 μm filter. SSAO activity was determined by both radioenzymatic and fluorometric methods as previously described (Yu, 1986, In Neuromethod; neutransmitter enzymes. (Eds: Boulton AA, Baker G & Yu PH).

CPA 47 endothelial cells (ATCC CRL-1733) derived from the pulmonary vein of normal young calf were grown in Dulbecco's modified Eagle's medium and Ham's nutrient mixture F12 with 15 % fetal bovine serum containing sodium bicarbonate (1.2 g/L), streptomycin (100 μg/mL), penicillin (100 unit/mL) and l-glutamine (20 mM) and maintained in a humidified atmosphere of 5 % CO_2 and 95% air at 37°C. Fresh medium was replaced twice a week. For subculture the medium was removed and 0.01 % EDTA and 0.1 % collagenase were added, and the cells were then incubated at room temperature for ten min until cells detach. Fresh medium was then added, asperated and dispensed into new flasks. The subcultivation ratio was maintained at 1:3.

Endothelial cells were also obtained from human umbilical cord vein according to Jaffe et al (1973, J. Clin. Invest. 52:2745). The cord was severed from the placenta soon after birth and placed in a sterile container filled with Hank's balanced salt solution (HBSS). The vein was perfused with HBSS to wash out blood and followed by infusion of a digestion solution containing 0.2 % collagenase and 0.02 % elastase in HBSS and incubated at 37°C for 20 min. The solution containing the endothelial cells were flushed out from the vein and the endothelial cells were collected by centrifugation at 3,000 g for 10 min. The pellets were washed once with HBSS and resuspended with Dulbecco's Modified Eagle's Medium (DMEM) containing 20 % fetal bovine serum, penicillin (200 units/mL) streptomycin (200μg/mL) and glutamine (20 mM). The conditions for maintaining cell growth were identical to those described above for the cell line.

Quantitative assessment of the cytotoxic effects of methylamine and SSAO were conducted on 96-wells plates. The endothelial cells ($6x10^3$ cells/well) were subcultured and incubated for 24 hours, then methylamine, formaldehyde, hydrogen

peroxide and SSAO (0.56 nmoles/min/mL) alone or in combination, in the presence or absence of the SSAO inhibitors semicarbazide ($2x10^{-4}$M) and MDL 72,974A ($1x10^{-7}$M) were added and further incubated for 24 hours. Cell survival was then examined using a MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] dye exclusion assay and the estimation of DNA synthesis [i.e. 3H-thymidine incorporation method] was according to Branch and Guibert (1986, J. Tissue Culture Methods 10:101). MTT stock solution was prepared by dissolving 5 mg/mL in PBS which was then filtered to sterilize and remove a small amount of insoluble residue. MTT solution was prepared by dilution of the MTT stock with medium of 1% serum. The media in the 96-wells were removed and 50 µL MTT solution was added; the plates were then incubated at 37oC for 4 h. Acidified isopropanol (100 µL of 0.04 N HCl in isopropanol) was added and mixed thoroughly to dissolve the dark blue crystals. After 10 min at room temperature the plates were read on a UVmax microplate reader (Molecular Device, Menlo Park, CA, USA) using a wavelength of 590 nm. For assessment of DNA synthesis 20 µL of DMEM containing 0.5 µCi [3H]-thymidine was added to each well and incubated for 4 h. After removal of the medium the cells were washed with 0.1 % Triton-X100 (in PBS) and collected with a cell harvester (Brandel, Gaithersburg, MD, USA). Radioactivity was estimated in a scintillation counter (Beckman LS 500TD, Irvine, CA, USA). Morphological changes were assessed directly examined under a inverse light microscope.

<u>SSAO activities in cardiovascular tissues of different species</u>

SSAO activities in bovine and rat aortae, in human umbilical arteries and in bovine serum amine oxidase (BSAO) towards several substrates, i.e. benzylamine, methylamine, pentylamine, tyramine and

putrecine, were assessed. Each of the enzyme preparations was pretreated with clorgyline ($1x10^{-4}M$) to ensure that both MAO-A and MAO-B were inhibited. The substrate preference of the enzymes was assessed by measuring the SSAO activity towards different substrates in comparison to that of benzylamine, a typical SSAO substrate. The relative activities of enzymes from the different sources varied considerably. Purified BSAO appears to be active towards pentylamine as well as benzylamine, but it is relatively inactive towards methylamine. SSAO from bovine aorta seems to exhibit weak activity towards methylamine and tyramine. SSAOs from rat aorta and human umbilical arteries, however, readily deaminate methylamine. Methylamine appears to be a better substrate than benzylamine for rat aorta and umbilical artery SSAOs.

Effect of methylamine and SSAO on CPA 47 endothelial cells

The deamination of methylamine proved to be toxic towards endothelial cells (CPA 47, ATCC CRL-1733). Survival of the endothelial cells was assessed using MTT dye exclusion and cell proliferation by thymidine incorporation methods (Table 1). Methylamine alone did not significantly affect the viability of the cells, except at relatively higher concentrations, i.e. 10 mM, neither did SSAO alone. SSAO in the presence of methylamine, however, caused severe toxic effects on the cultured endothelial cells. The ED50 values were estimated to be $5\ x10^{-4}M$ and $1x10^{-5}M$ for the MTT assay and DNA synthesis method respectively. The SSAO inhibitors semicarbazide and MDL-72974A were able to prevent these toxic effects. Semicarbazide is a weak SSAO inhibitor, so relatively high concentrations ($2x10^{-4}M$) were required to prevent partially the death of some of the cells. This is a problem as high concentrations of semicarbazides are difficult to administer in vivo because of their toxicity.

MDL-72974A, a much more potent SSAO inhibitor exhibited much better protection against the methylamine-SSAO mediated toxicity and this occurred at much lower concentration ($1x10^{-7}M$).

TABLE 1

Cytotoxic effect of the oxidative deamination of methylamine on CPA 47 endothelial cell and the protection of such effect by SSAO inhibitors

		Addition of			
	Methyl-amine	Control	SSAO	SSAO + MDL	SSAO + SC
MTT assay	$1x10^{-2}M$	.283±.037	.071±.002	.458±.031	.090±.002
(OD)	$5x10^{-3}M$	.300±.032	.084±.004	.419±.033	.126±.013
	$1x10^{-3}M$	.283±.001	.102±.001	.388±.022	.304±.012
	$5x10^{-4}M$	.331±.001	.251±.022	.388±.004	.411±.014
	$1x10^{-4}M$	.365±.056	.345±.015	.403±.013	.402±.016
	$1x10^{-5}M$	.342±.012	.437±.033	.395±.014	.412±.012
DNA	$1x10^{-2}M$		1203±231	25959±1671	305±67
Synth.	$5x10^{-3}M$		1059±264	55908±2976	435±75
(dpm)	$1x10^{-3}M$		1330±226	58897±5033	512±163
	$5x10^{-4}M$		1889±92	58789±4878	47586±302
	$1x10^{-4}M$		7471±667	57289±5404	56153±4562
	$1x10^{-5}M$		6225±3871	60922±2937	60927±6503

The cells, 24 hours after subculture ($6x10^3$ cells/well) on 96-wells plates were used. The cultured

endothelial cells were incubated with methylamine at different concentrations in the presence or absence of SSAO (0.56 nmoles/min/mL). MDL-72974A (MDL) ($1x10^{-7}$M) and semicarbazide (SC) ($1x10^{-4}$M) were added 30 min. before methylamine to inhibit SSAO activity. Twenty-four hours after treatment the effect on the survival of the cells was estimated using either MTT dye exclusion or [^{3}H]-thymidine incorporation. Results are mean ± S.D. (n=4).

TABLE 2

Cytotoxic effect of the oxidative deamination of methylamine on primary cultured endothelial cell obtained from human umbilical vein and the protection of such effect by SSAO inhibitors

		Addition of		
	Methyl-amine	Control	SSAO	SSAO + MDL
MTT assay	$1x10^{-2}M$	.495±.028	.016±.000	.430±.019
(OD)	$5x10^{-3}M$	.494±.036	.015±.000	.507±.044
	$1x10^{-3}M$	.491±.035	.036±.006	.466±.042
	$5x10^{-4}M$	.452±.040	.288±.016	.448±.032
	$1x10^{-4}M$	.459±.021	.384±.036	.444±.036
	$5x10^{-5}M$	.495±.024	.455±.026	.441±.028
	$1x10^{-5}M$	.500±.032	.486±.015	.454±.014
	$1x10^{-6}M$	.468±.047	.486±.047	.486±.047
DNA	$1x10^{-2}M$	1583±293	126±10	4911±159
Synth.	$5x10^{-3}M$	1854±409	257±13	5498±279
(dpm)	$1x10^{-3}M$	1614±278	237±18	5331±327
	$5x10^{-4}M$	2645±242	449±95	6668±677
	$1x10^{-4}M$	2680±269	919±123	7131±773
	$5x10^{-5}M$	3427±490	3948±279	6929±710
	$1x10^{-5}M$	3658±690	5829±828	6816±494
	$1x10^{-6}M$	3466±261	3297±321	6482±217

The cells, 24 hours after subculture ($6x10^3$ cells/well) on 96-wells plates were used. The cultured endothelial cells were incubated with methylamine at different concentrations in the presence or absence of SSAO (0.56 nmoles/min/mL). MDL-72974A (MDL) ($1x10^{-7}$M) and semicarbazide (SC) ($1x10^{-4}$M) were added 30 min. before methylamine to inhibit SSAO activity. Twenty-four hours after treatment the effect on the survival of the cells was estimated using either MTT dye exclusion or [^{3}H]-thymidine incorporation. Results are mean ± S.D. (n=4).

Effect of methylamine and SSAO on primary culture endothelial cells derived from human umbilical vein

Deamination of methylamine was also found to be toxic towards the primary cultured endothelial cells derived from the human umbilical vein. The survival of these endothelial cells was shown to be quite similar to that of the endothelial cell line (CPA 47). As can be seen from Table 2, the viability of the cells was not affected by methylamine or SSAO alone, but SSAO together with methylamine caused substantial damage to the cells. The ED_{50} values were estimated to be $2x10^{-4}$ M and $>1x10^{-5}$ M according to the MTT and DNA synthesis assays respectively. The inclusion of the SSAO inhibitor MDL-72974A also prevented the toxicity of human endothelial cells seen in the presence of SSAO and high concentrations of methylamine. It is worthy of note that the crude SSAO preparations itself, from umbilical veins, exhibited a stimulatory effect on DNA synthesis in both human and bovine endothelial cells. It did not, however, effect the dehydrogenase content as revealed by the MTT method.

Morphological effects of methylamine and SSAO on CPA 47 endothelial cells derived from bovine pulmonary vein

Exposure to methylamine in the presence of SSAO also caused morphological changes such as the

disappearance of pseudopodia, vacuolization, and contracture of the cells. Neither methylamine (up to $1x10^{-3}M$) nor SSAO (0.56 nmoles/min/ml) alone induced these morphologic changes. The SSAO inhibitor MDL-72974A ($1x10^{-7}M$) prevented the morphological changes seen with methylamine plus SSAO. The methylamine-SSAO induced cytotoxicity towards the endothelial cells is time dependent. In the presence of methylamine ($1x10^{-3}M$) and SSAO (0.56 nmoles/min/m) the morphological changes were readily visible after one hour. Disruption of intercellular junctions and increases in vacuole formations increased with time until cell death, which occurred about after 20 hours of treatment, namely, the cells were completely detached from the plates.

Effect of methylamine and human serum on endothelial cells (CPA 47)

A small amount of SSAO is known to circulate in normal human blood. Serum obtained from a normal human subject was therefore included in the culture media in order to examine whether or not such serum SSAO is involved in the deamination of methylamine and thus capable of inducing cellular damage. As can be seen in Table 3, methylamine or serum alone does not affect the cell viability. In the presence of both human serum and methylamine, however, the survival of the endothelial cells was reduced. The ED_{50} was estimated to be $1x10^{-3}M$. Since human serum SSAO activity is relatively lower than the isolated umbilical SSAO, it is not surprising that it is somewhat less effective in causing damage to the endothelial cells. As was the case with earlier experiments the inclusion of MDL-72974A prevents the appearance of any cytotoxic effects except at high concentration of methylamine.

TABLE 3

Cytotoxic effect of methylamine in the presence of human serum on endothelial cells from human umbilical vein and the protection of such effect by SSAO inhibitors

		Addition of			
	Methyl-amine	Control	SSAO	Serum	Serum + MDL
MTT assay	$1x10^{-2}M$	.365±.041	.000±.000	.000±.000	.122±.023
(OD)	$5x10^{-3}M$	.327±.035	.000±.000	.000±.000	.267±.040
	$1x10^{-3}M$	.362±.036	.000±.000	.006±.000	.325±.033
	$5x10^{-4}M$	.328±.026	.000±.000	.105±.027	.374±.025
	$1x10^{-4}M$	.335±.030	.152±.008	.305±.023	.368±.016
	$5x10^{-5}M$	.347±.032	.229±.020	.312±.038	.368±.041
	$1x10^{-5}M$	.360±.022	.318±.030	.332±.036	.360±.039
	$1x10^{-6}M$	.356±.041	.344±014	.344±.014	.349±.019

The cells, 24 hours after subculture ($6x10^3$ cells/well) on 96-wells plates were used. The cultured endothelial cells were incubated with methylamine at different concentrations in the presence or absence of SSAO (0.56 nmoles/min/mL). MDL-72974A (MDL) ($1x10^{-7}M$) and semicarbazide (SC) ($1x10^{-4}M$) were added 30 min. before methylamine to inhibit SSAO activity. Twenty-four hours after treatment the effect on the survival of the cells was estimated using either MTT dye exclusion. Results are mean ± S.D. (n=4).

Effect of methylamine in vivo; involvement of SSAO in the generation of endogenous formaldehyde, which binds to tissues irreversibly

In order to assess whether or not deamination of methylamine occurs in vivo and whether the degradation product formaldehyde indeed harmfully interacts with tissue components, [^{14}C]-methylamine was administered to mice and it was found that a large quantity of the radioactivity resides in various tissues (see Table 4). Interestingly, the residual radioactivity in the tissues remains very high for a very long time after administration of [^{14}C]-methylamine. For example, over 80% of residual radioactivity was still detected in the heart and brain on day 5 in comparison to day one after i.v. injection of [^{14}C]-methylamine. Free methylamine should be all washed out after such a time period. In other tissues (i.e. spleen and liver) the residual radioactivity was reduced with time. This long lasting residual radioactivity in tissues suggests that formaldehyde (the deaminated product of methylamine) is irreversibly linked with some tissue component.

In animals pretreated with SSAO inhibitor MDL-72974A, more than 80% of the irreversible binding of radioactivity in the tissues were prevented. It has also been found that semicarbazide, another SSAO inhibitor, exhibited a very similar effect as that of MDL-72974A in blocking the formation of irreversible residuals in the tissues. It is clear that when SSAO activity is inhibited, methylamine cannot be metabolized to formaldehyde and would be subject to wash out. If methylamine is deaminated, the oxidative product formaldehyde quickly reacts with tissue components. Irreversible interactions likely occur under pathological conditions, when deamination of methylamine is excessive. In this context, cytotoxic effects are expected. For example, this may cause

endothelial lesion and subsequently atherosclerosis, or pains at joint tissues, which is rich in SSAO (Lyles and Berti, 1987, Pharmacology Toxicology (supplement), Vol. 60, p. 33).

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TABLE 4

Residual radioactivities in different mouse tissues following i.v. administration of [^{14}C]-methylamine

Tissues	Residual radioactivities (dpm/g fresh tissue)					
	24 h		48h		120 h	
	Saline	MDL	Saline	MDL	Saline	MDL
Kidney	107292±7595	32439±2679	69446±9436	33439±3234	51763±2159	13182±520
Heart	65693±5482	13773±481	63557±4722	14653±1019	52666±2387	14858±1705
Liver	81853±6157	23727±1026	49761±7258	18715±3357	31700±3835	6889±1699
Spleen	125844±11330	22584±2765	63609±9756	25077±2502	35601±2307	11578±238
Brain	17446±199	6068±1103	16105±1372	4810±271	15408±783	5123±304
Serum	13305±912	4533±854	9368±1325	3039±402	6583±953	2436±34

Male Swiss white mice (25-30g) were preated with saline or SSAO inhibitor MDL [MDL-72974A, (E)-2-(4-fluorophenethyl)-3-fluoroallylamine], followed by an i.v. injection of [^{14}C]-methylamine (1 mg/K, 2 μCi/mouse). Different tissues were dissected out after 24, 48 and 120 hours. Aliquots of the tissues were incubated with a tissue solubilizer (Aquasol, NEN) overnight and the radioactivity was assessed in a liquid scintillation counter. Results are means ± SE (n = 3).

Effect of streptozotocin (STZ) on the urinary excretion of methylamine

Urinary methylamine levels have also been found in STZ-induced diabetic mice. As can be seen in Table 5, the urinary methylamine levels are not significantly increased in the STZ-induced diabetic rats. When the rats were treated with SSAO inhibitor MDL-72974A, urinary methylamine increased significantly. There was an additional increase of urinary methylamine in the diabetic rats treated with MDL-72974A. These results suggest that STZ does induce the synthesis of methylamine. The increase of methylamine was not detectable in the case of treatment with STZ alone, because methylamine would be quickly metabolized by SSAO. If SSAO is blocked, however, the increase of methylamine then becomes apparent.

TABLE 5

Effect of streptozotocin (STZ) and MDL-72974A on the rat urinary excretion of methylamine

μg Methylamine/mg creatinine

	First week	3rd week
Controls	46.1±7.7	30.2±1.4
STZ	48.4±8.0	37.0±5.6
MDL	61.2±5.8[a]	50.4±10.1[a]
MDL + STZ	82.7±10.3[a,b]	61.8±6.8[a]

Diabetic rats were induced by single i.p. administration of STZ (60 mg/Kg, i.p.). SSAO activity was inhibited by daily i.p. injections of inhibitor MDL-72974A (2 mg/Kg). Methylamine was prepurified through a Sep-Pak of C-18 cartridge, derivatized with OPA and analyzed by HPLC with fluorometric detection.

a statistically significant from the saline controls

b statistically significant different between the MDL groups.

Results are mean ± (n = 5).

DISCUSSION

Methylamine has been found to be readily deaminated by SSAO to produce formaldehyde. Methylamine has been detected in different mammalian tissues and in blood and is excreted in relatively large quantities by humans. Methylamine can be derived from the diet, gut bacterial degradation of dietary

precursors, and from various metabolic pathways for the degradation of creatinine, sarcosine and adrenaline. Normal circulating and urinary concentrations of methylamine are increased in certain physiological and pathological (i.e. diabetes, uraemia) circumstances. SSAO, which presents predominantly in the vascular and cartilage tissues, exhibits a reasonably high affinity for methylamine with a Km value of 1 x 10-4 M. The urinary excretion of methylamine is increased in rats treated with SSAO inhibitors. Methylamine is therefore an endogenous substrate.

Cultured endothelial cells derived from both bovine and human blood vessels possess negligible SSAO activity. Calf serum, which is used in culture media, contains small amounts of deaminating activity towards benzylamine (probably due to BSAO); it exhibits quite low activity towards methylamine, however. This explains why methylamine by itself does not exhibit toxicity towards the endothelial cells as assessed by MTT dye exclusion measurement. When SSAO isolated from human umbilical arteries is included in the cell culture media, toxicity to methylamine is induced. This toxic effect of deamination of methylamine on endothelial cells is even more pronounced, when DNA synthesis ([^{3}H]-thymidine incorporation study) is assessed. DNA synthesis in the endothelial cells was observed to be enhanced by the addition of the umbilical SSAO preparation, which perhaps indicate the presence of some growth factors. The methylamine-SSAO mediated cytotoxicity is a result of deamination of methylamine. This conclusion is further confirmed by the ability of the SSAO inhibitor MDL-72974A to prevent completely this methylamine-SSAO induced toxicity. Semicarbazide, the classical SSAO inhibitor, also appears be able to protect endothelial cells against these toxic effects, but it is less effective than MDL-72974A.

If the deamination of methylamine is uncontrolled, it might cause cellular damage in the blood vessels. This has been proved to be the case for some vascular disorders such as uraemia and diabetes. In the uraemia patients serum methylamine is increased drastically (Baba et al., 1984, Clin. Chim. Acta. 136:49-56). In the diabetic patients, the serum SSAO activity is significantly increased (Nilssson et al., 1968, Acta Med. Scand. 184:105-108; McEwen and Castell, 1067, J. Lab. Clin. Med. 70:30-47; Tryding et al., 1969, Scand. J. Lab. Invest. 70:36-47). Elevation of serum SSAO activity has also been detected in toxin-induced animals (Hayes and Clarke, 1990, Res. Comm. Chem. Pathol. Pharmacol. 69:71-83; Elliot et al., 1991, Res. Vet. Sci. 50:334-339). It is also probable that other disorders, such as gout and arthritis, where the joint tissues rich of SSAO, abnormal deamination of methylamine might be involved. SSAO inhibitors can act to control methylamine deamination in vivo.

Both formaldehyde and hydrogen peroxide are formed during the deamination of methylamine and both products are known to be toxic. Formaldehyde is capable of conjugating with proteins and nucleic acids, to modify their chemical structures and thus change or inactivate their functions. Hydrogen peroxide, a strong oxidative agent, is involved in various oxidative reactions and can itself be transformed into an hydroxyl free radical in the presence of certain metal ions. If detoxification of these products were to be deficient in vivo, they could cause tissue damage. It is interesting to know whether formaldehyde or hydrogen peroxide is responsible for causing the cytotoxicity of the endothelial cells seen in this present study. An approximately 30-fold higher concentration of hydrogen peroxide , however, is required to induce endothelial damage similar to that seen with formaldehyde. This suggests that the

toxicity induced by methylamine deamination is due primarily to the production of formaldehyde rather than hydrogen peroxide.

It has been demonstrated that SSAO-mediated conversion of methylamine to formaldehyde occurs in the blood or the vascular tissues. Vascular disorders could be,therefore, induced where SSAO is located. Blood methylamine levels have been estimated to be 2.3 μg/100 mL (7.4x10-7M) in healthy control subjects and 26 μg/100 mL (8x10-6M) in patients with chronic renal failure before haemodialysis (Baba et al, 1984, Clin. Chim. Acta. 136:49). The methylamine concentration in such patients is thus quite comparable to the concentrations used in the above experiments to cause damage to endothelial cells. Vascular problems are known to be associated with patients exhibiting renal failure and dysfunction.

The amount of SSAO in blood is another important factor which might determine whether or not deamination of methylamine can be increased to the point that it can become toxic in certain pathological conditions. It has been found that in blood from healthy subjects SSAO activity is about 0.7 nmoles/min/mL. This is also quite similar to the amount of SSAO (0.56 nmoles/min/mL) added to the above described cultured endothelial cell studies.

Repeated endothelial cell injury has been suggested as an initiating factor in atherosclerosis. It has been found that the biotransformation of methylamine to formaldehyde plays an important role in the initiation of endothelial injury. The formaldehyde produced links covalently with the endothelial cell surface and subsequently damages the cells and causes vascular disorders. When radio-isotopically labelled methylamine was administered to the rats, long lasting residual radio-isotope activities were found in the tissues (Table 4). Methylamine is metabolized by SSAO

in vivo and the labelled formaldehyde product interacts with tissue components and forming irreversible linkage. This is also supported by the finding that an increased metabolism of methylamine indeed occurs in STZ-induced diabetic rats (see Table 5). Reduction of deamination of methylamine by blocking SSAO activity with specific inhibitor therefore reduces the risk of SSAO related vascular damage.

Claims to the invention follow.

CLAIMS

1. A method for preventing endothelium damage in mammals, said method comprising administering to a patient in need thereof an effective amount of a SSAO inhibitor for the purpose of preventing endothelium damage in mammals.

2. A method according to claim 1, wherein said endothelium damage is a cardiovascular disorder associated to diabetes and uraemia in mammals.

3. A method according to claim 1, wherein said SSAO inhibitor is selected from fluoroallylamine, semicarbazides, hydrazides and derivatives thereof.

4. A method according to claim 3, wherein said SSAO inhibitor is (E)-2-(4-fluorophenethyl)-3-fluoroallylamine HCl.

5. Use of a SSAO inhibitor for preventing endothelium damage in mammals.

6. Use according to claim 5, wherein said endothelium damage is a cardiovascular disorder associated with diabetes and uraemia.

7. Use according to claim 5, wherein said SSAO inhibitor is selected from fluoroallylamines, semicarbazides, hydrazides and functional derivatives thereof.

8. Use according to claim 7, wherein said SSAO inhibitor is (E)-2-(4-fluorophenethyl)-3-fluoroallylamine HCl.

9. Use of a SSAO inhibitor in the preparation of a medicament for preventing endothelium damage in mammals.

10. Use according to claim 9, wherein said endothelium damage is a cardiovascular disorder associated with diabetes and uraemia.

11. Use according to claim 9, wherein said SSAO inhibitor is selected from fluoroallylamines,

semicarbazides, hydrazides and functional derivatives thereof.

12. Use according to claim 11, wherein said SSAO inhibitor is (E)-2-(4-fluorophenethyl)-3-fluoroallylamine HCl.

13. A method for alleviating pain associated with gout and arthritis, said method comprising administering to a patient in need thereof an effective amount of a SSAO inhibitor for the purpose of alleviating pain associated with gout and arthritis.

14. A method according to claim 13, wherein said SSAO inhibitor is selected from fluoroallylamine, semicarbazides, hydrazides and derivatives thereof.

15. A method according to claim 14, wherein said SSAO inhibitor is (E)-2-(4-fluorophenethyl)-3-fluoroallylamine HCl.

16. Use of a SSAO inhibitor for alleviating pain associated with gout and arthritis.

17. Use according to claim 16, wherein said SSAO inhibitor is selected from fluoroallylamines, semicarbazides, hydrazides and functional derivatives thereof.

18. Use according to claim 17, wherein said SSAO inhibitor is (E)-2-(4-fluorophenethyl)-3-fluoroallylamine HCl.

19. Use of a SSAO inhibitor in the preparation of a medicament for alleviating pain associated with gout and arthritis.

20. Use according to claim 19, wherein said SSAO inhibitor is selected from fluoroallylamines, semicarbazides, hydrazides and functional derivatives thereof.

21. Use according to claim 20, wherein said SSAO inhibitor is (E)-2-(4-fluorophenethyl)-3-fluoroallylamine HCl.

INTERNATIONAL SEARCH REPORT

International Application No PCT/CA 93/00198

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 A61K31/135; A61K31/175; A61K31/15

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	A61K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
P,X	DIABETES vol. 42, no. 2, April 1993, pages 594 - 603 P. YU ET AL. 'Oxidative deamination of methylamine by semicarbazide-sensitive amine oxidase leads to cytotoxic damage in endothelial cells' see the whole document	1-21
X	CIRCULATION RESEARCH vol. 66, no. 1, January 1990, pages 249 - 252 P-J- BOOR ET AL. 'A role for a new vascular enzyme in the metabolism of xenobiotic amines' see the whole document	1-3,5-7, 9-11

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° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
23 JULY 1993	12. 08. 93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	FOERSTER W.K.

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

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Y	TOXICOLOGY vol. 73, no. 2, 1992, pages 251 - 258 P.J. BOOR ET AL. 'Methylamine metabolism to formaldehyde by vascular semicarbazide-sensitive amine oxidase' see the whole document	1-12
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A	J. AUTON. PHARMACOL. vol. 11, no. 5, October 1991, pages 323 - 335 J. ELLIOTT ET AL. 'Effect of benzylamine and its metabolites on the responses of the isolated perfused mesenteric arterial bed of the rat' see the whole document	1-21
A	EP,A,0 295 604 (MERRELL DOW PHARMACEUTICALS INC) 21 December 1988 see the whole document	1-21

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

CA 9300198
SA 73908

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on
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